Short communication

Detection and titration of bluetongue virus in *Culicoides* insect cell culture by an antigen-capture enzyme-linked immunosorbent assay

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Abstract

Bluetongue virus (BTV) infects sheep, cattle and other ruminants and is transmitted by *Culicoides* spp. of biting midges. Virus is typically isolated and characterized by infection of susceptible vertebrate cells that undergo detectable and measurable cytopathic effects. Cell lines derived from *C. sonorensis* may be useful for virus isolation and studies to better understand BTV replication in the insect vector. However, their use is hampered because BTV infection does not produce significant cytopathic effects in these insect cell cultures. Detection of virus replication in these cells typically requires co-cultivation with susceptible vertebrate cell culture. This report describes the use of an antigen-capture enzyme-linked immunosorbent assay (Ag-Cap ELISA) for direct detection and titration of BTV in cultures of a *Culicoides* cell line. This assay should facilitate use of this cell line for virus isolation, titration and studies of BTV replication.
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Bluetongue virus (BTV) is an *orbivirus* that infects sheep, cattle and other ruminants and is transmitted by *Culicoides* spp. of biting midges (Borden et al., 1971; Matthews, 1982; Price and Hardy, 1954). Vertebrate cell cultures that exhibit cytopathology following infection have been useful for BTV isolation and titration. This was first demonstrated in primary lamb kidney cell culture infected with egg-adapted strains of BTV (Haig et al., 1956). Subsequently, varying degrees of virus replication and cytopathology have been shown in numerous vertebrate cell lines following infection with BTV (Fernandes, 1959; Wechsler and McHolland, 1988). One cell line, bovine pulmonary artery endothelium (CPAE), was shown to be particularly sensitive to detection of BTV from infected sheep blood (Wechsler and Luedke, 1991). Plaque formation (gross, visible cytopathology) in the cell culture monolayer was first demonstrated in mouse fibroblast cell culture following infection with egg/cell culture adapted BTV (Howell et al., 1967). This useful technique has been expanded to other vertebrate cell types (Wechsler and McHolland, 1988).

Several cell lines have been derived from *C. sonorensis* (McHolland and Mecham, 2003; Wechsler et al., 1989; Wechsler et al., 1991). These cell lines have provided a useful resource for research issues relevant to the insect vector and replication of BTV (Mertens et al., 1996; Mortola et al., 2004; Nunamaker et al., 1999; Xu et al., 1997). Bluetongue virus replicates in the *Culicoides* insect cells; but, since there are few observable cytopathic effects, virus is detected and titrated by co-cultivation of the infected insect cells or cellular material with susceptible vertebrate cell culture (McHolland and Mecham, 2003; Mertens et al., 1996; Wechsler et al., 1989). Immunoperoxidase staining can be used to detect virus in the insect cells (Wechsler et al., 1991). However, this technique, which requires the use of a microscope to score the results, suffers from a degree of subjectivity, is not easily quantifiable, and has not been used for titrating BTV. Titration of Dengue virus in *A. albopictus* (C6/36) cells by immunofluorescence staining in microtiter plates has been described (Schoepp and Beaty, 1984). Attempts, in this laboratory, to use immunofluorescence for detection and titration of BTV in *Culicoides* cell culture have not been very successful because of poor sensitivity and inconsistent results (unpublished data). An antigen-capture enzyme-linked immunosorbent assay (Ag-Cap ELISA) was previously used to

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detect and quantitate BTV from sheep blood following amplification in Vero-MARU (VM) cell culture (Mecham, 1993). In the current study, the Ag-Cap ELISA was adapted for detection and titration of BTV in *Culicoides* cell culture. This direct, consistent and easily quantifiable assay should make the *Culicoides* cell lines more attractive for both research and diagnostic applications.

The KC cell line, derived from *C. sonorensis* insects (Wechsler et al., 1991) and VM cells were used in this study. Bluetongue virus serotypes 10 and 11 (BTV-10 and BTV-11), and epizootic hemorrhagic disease virus serotype 2 (EHDV-2) stocks were produced in baby hamster kidney (BHK-21) cells. Following infection, the cells were disrupted by sonication, centrifuged to remove cellular debris, and the supernatant material saved for titration in VM and KC cell cultures.

The stock virus preparations were titrated by visualization of cytopathology in VM cell culture as previously described (Mecham, 1993). Briefly, quadruplicate dilutions of virus were made in 96-well microtiter plates in a final volume of 100 μl/well. One hundred microliters of a suspension of VM cells ($\sim 7 \times 10^5$/ml) was added to each well to produce a monolayer, and the microtiter plates were incubated at 34 °C. Growth of BTV in VM has been shown to be optimal at 34 °C (Samal et al., 1985). At 6 and 7 days after infection, cytopathic effect was visualized and scored after fixing and staining the cell monolayer in the microtiter plates with 10% formalin and crystal violet. Virus titers were determined using the Spearman–Karber method for calculation of 50% cell culture infective doses (CCID$_{50}$) (Ballew, 1986).

The stock virus preparations were titrated in KC cell culture using a modified version of the previously described Ag-Cap ELISA (Mecham, 1993). Briefly, quadruplicate dilutions of virus were made in 96-well microtiter plates in a final volume of 100 μl/well. One hundred microliters of a suspension of KC cells ($\sim 1.8 \times 10^6$/ml) was then added to each well to create a monolayer, and the plates were incubated at 34 °C. At daily intervals after infection, a single 96-well plate was removed from incubation, frozen overnight at −70 °C, and then rapidly thawed to disrupt the cells. One hundred microliter aliquots of this freeze/thawed material were removed from the wells of the plate and added to the corresponding wells of a 96-well microtiter plate to which rabbit polyclonal anti-BTV serum had been adsorbed. A mouse monoclonal antibody to BTV (1AA4.E4) (Mecham et al., 1990) was then allowed to react with any captured virus/viral antigen and detected by the sequential addition of biotinylated goat anti-mouse, streptavidin-conjugated peroxidase and *o*-phenylenediamine. The amount of color development was determined by spectrophotometer measurement at 492 nm. Wells with an absorbance of two or more times the background of uninfected cells were scored as positive and used to calculate 50% cell culture infective doses (CCID$_{50}$) by the Spearman–Karber method (CCID$_{50}$) (Ballew, 1986).

The results of BTV and EHDV titration in VM cell culture by staining for cytopathology and in KC cell culture by the Ag-Cap ELISA are shown in Fig. 1. Virus titers calculated at 6 and 7 days
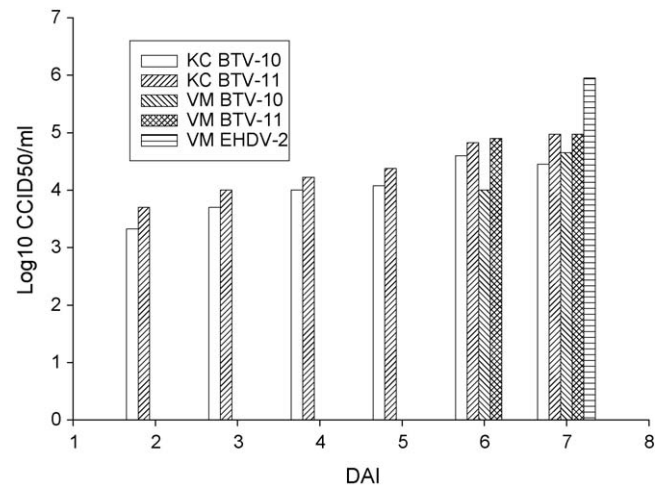

Fig. 1. Bluetongue virus serotypes 10 and 11 (BTV-10 and BTV-11) and epizootic hemorrhagic disease virus serotype 2 (EHDV-2) were titrated in VM cell culture by staining for cytopathic effect and in KC cell culture by the Ag-Cap ELISA. Virus titers were determined using the Spearman–Karber method for calculation of 50% cell culture infective doses (CCID$_{50}$) (Ballew, 1986). Infected cell cultures were processed at the indicated days after infection (DAI).

after infection (the standard time points for staining infected VM cells) were similar in both KC and VM cell cultures for both serotypes of BTV. In addition, the Ag-Cap ELISA was able to detect virus in the KC cells as early as 2 days after infection. The calculated virus titers in KC cell culture increased through day 6 after infection. The Ag-Cap ELISA did not detect the related EHDV-2 in infected KC cell culture, even though $\sim$6 logs of virus/ml was present in the stock sample as determined by titration in VM cell culture (Fig. 1).

Initially, titration of BTV in KC cell culture was attempted using untreated cell culture supernatant material as the source of virus/viral antigen for the Ag-Cap ELISA, as previously described for detection of BTV from sheep blood following amplification in VM cell culture (Mecham, 1993). However, using that strategy, virus was often not detected, or detected in only one or two wells of the 96-well plates, 6 or 7 days after infection. Therefore, it was impossible to do any significant virus titer calculations using the Spearman–Karber method. Modification of the procedure to include a freeze/thaw step resulted in reproducible adequate numbers of positive wells for titer calculations. As has been previously noted, *Culicoides* cell culture does not undergo notable cytopathology following infection with BTV (McHolland and Mecham, 2003); therefore, disruption of the cells is necessary for release of virus and viral antigen for detection by the Ag-Cap ELISA.

This is the first description of direct virus titration in *Culicoides* cell culture without co-cultivation with susceptible vertebrate cells. The procedure is objective and quantifiable. Since the KC cell line used in this study was derived from the vector for BTV, direct detection and titration of virus in these cells has obvious value in various research questions. In addition, this system may be particularly useful for the isolation and characterization of virus from field collected insects without the need for adaptation to vertebrate cell culture.

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